

Evaluation of Genetic Immunization Adjuvants to Improve the Effectiveness of a Human Immunodeficiency Virus Type 2 (HIV-2) Envelope DNA Vaccine

CHRISTOPHER P. LOCHER, STEPHANIE A. WITT, BRITTANY M. ASHLOCK, and JAY A. LEVY

ABSTRACT

In an effort to develop a more effective genetic immunization strategy for HIV, we developed an HIV-2 *env* DNA vaccine and evaluated three adjuvant formulations. The gp140 gene from HIV-2_{UC2} was synthesized using mammalian codons and cloned into a plasmid vector that expresses eukaryotic genes at high levels. We found that after three immunizations in mice, a novel cationic liposome formulation (Vaxfectin) was superior at inducing systemic and mucosal antibody responses compared to a naked DNA, a controlled release device (an Alzet minipump) and polysaccharide microparticles made from chitosan ($P = 0.027$). Vaxfectin also induced higher levels of systemic antibodies for each isotype and IgG subclass as well as levels of HIV-2-specific mucosal IgA ($P = 0.034$). When different routes of immunization were used with the Vaxfectin formulation, gp140-specific systemic antibody responses were highest by the intradermal route, mucosal antibody responses were highest by the intramuscular route, while the intranasal route was the least effective. These results suggest that this cationic liposome formulation is an important adjuvant to improve the effectiveness of genetic immunization strategies for AIDS, and that multiple routes of immunization should be employed for optimal efficacy for HIV vaccine candidates.

INTRODUCTION

MORE EFFECTIVE VACCINE CANDIDATES for HIV need to be developed to induce robust cellular-mediated and neutralizing antibody-immune responses capable of recognizing multiple epitopes (Letvin *et al.*, 2002; Robinson 2002). To achieve this goal, genetic immunization with DNA has been an encouraging strategy because memory, effector, and humoral immune responses are elicited (Ulmer *et al.*, 1996). DNA vaccines appear to be stable, safe, and affordable, and therefore, practical to use in the developing world (Hasan *et al.*, 1999). Nevertheless, since DNA vaccines are rapidly degraded after they are injected into the body and induce marginal immune responses in humans, new adjuvants and delivery systems are needed as well as improved prime-boost strategies (McKenzie *et al.*, 2001; O'Hagan and Lavelle, 2002). In this study, we compared different DNA adjuvant formulations in mice to improve the delivery of our HIV-2 gp140 vaccine. These adjuvant formulations were designed to protect the DNA vaccine from degradation and to facilitate stronger immune responses rather

than using a naked DNA vaccine alone. We compared cationic liposomes (Vaxfectin), an osmotic minipump (Alzet), and polysaccharide microspheres made from chitosan.

MATERIALS AND METHODS

The DNA vaccine constructs were developed based on the gene sequences of the gp140 envelope from the HIV-2_{UC2} isolate (Barnett *et al.*, 1996). The gene codons of the DNA vaccine constructs were synthesized for increased mammalian (optimized) expression (Haas *et al.*, 1996; zur Megede *et al.*, 2000), cloned into pND-14 (a generous gift from Dr. Gary Rhodes, University of California, Davis; Locher *et al.*, 2002) and endotoxin-free plasmid DNA preparations were prepared using anion exchange chromatography (Giga Endotoxin-free Purification kit, Qiagen, Chatsworth, CA).

For the cationic liposome formulation we used two lipids: (+/-)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(myristoyloxy)-1-propanaminium bromide (VC 1052) and diphy-

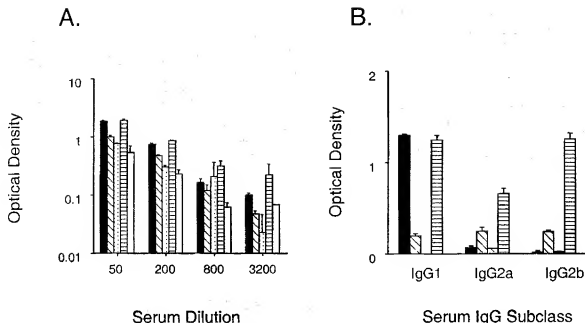


FIG. 1. HIV-2 gp140-specific mean end-point ELISA titers in mice immunized with various DNA vaccine formulations (A). Serum from mice immunized with naked DNA (solid bar), an Alzet miniosmotic pump (striped bar), Chitosan (stippled bar), Vaxfectin (horizontal lines; $P = 0.027$) and negative control samples (open bar) were evaluated using samples taken 28 days after the third DNA immunization by the intradermal route. The end-point ELISA titer was defined as an optical density value of 0.2. Optical density values for IgG-specific subclasses in serum samples diluted 1:100 in PBS (B). Samples from control-immunized mice were negative for IgG subclass determination.

tanoylphosphatidyl-ethanolamine (DpyPE), also known as Vaxfectin (a gift from Dr. Carl J. Wheeler, Vical, San Diego, CA) as previously described (Hartikka *et al.*, 2001). The final DNA to Vaxfectin molar ratio was 4:1, and was administered within 1 h of complexation. A miniosmotic pump (Alzet Model 2004, Alza, Palo Alto, CA) was used to deliver the HIV-2_{UC2} env plasmid DNA vaccine over a period of 28 days, and holds a total volume of 200 μ l. Prior to its evaluation as a DNA vaccine delivery device, we found that the DNA vaccine did not adhere to the osmotic minipump after 1 month at 37°C, since the DNA concentration remained stable and intact, as determined by agarose gel electrophoresis and a spectrophotometer. We surgically implanted the osmotic minipump containing 40 μ g of the HIV-2_{UC2} env DNA vaccine by the subcutaneous route into a group of mice that received one priming immunization of 20 μ g of the HIV-2_{UC2} env DNA vaccine by the intradermal route. The polysaccharide microspheres were formulated with the HIV-2_{UC2} env plasmid DNA using chitosan (molecular weight 390 kDa, a gift from Dr. Everett Nichols, Vanson Chemicals, Redmond, WA) as previously described (Roy *et al.*, 1999). This method was previously shown to produce uniform particles having a diameter of 150–300 nm, and was administered within 1 h of complexation.

Groups of five female Balb/c mice, weighing approximately 25 g, were used for the DNA immunization studies. Each mouse was immunized with 20 μ g of either naked DNA or formulated DNA divided into two sites by the intradermal route at the base of the tail. The DNA vaccine dosage of 20 μ g had been diluted into a total volume of 100 and 50 μ l of the DNA/PBS solution was injected into each site. For intramuscular injections, DNA was delivered using insulin-gauge needles into the quadriceps

muscle. For intranasal immunizations, DNA was delivered into the nares of anesthetized mice using DNA diluted into 50 μ l of PBS. The mice were then boosted with the same amount of DNA by the same route twice at 28 day intervals. In the group that received the osmotic minipump, they were immunized once by the intradermal route and the osmotic minipump was then

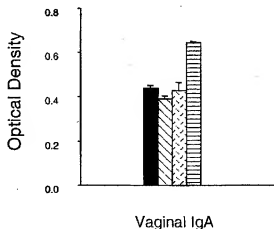


FIG. 2. Mucosal antibodies to HIV-2 gp140 in mice immunized with various DNA vaccine formulations as measured by IgA. Mice were immunized with naked DNA (solid bars), an Alzet mini osmotic pump (diagonal lines), Chitosan (stippled), and Vaxfectin (horizontal lines; $P = 0.034$) and the antibody levels were evaluated using vaginal washes diluted to 1:8 in phosphate buffer saline. Samples were taken 28 days after the third DNA immunization by the intradermal route.

implanted subcutaneously 1 month later. The osmotic minipump contained 40 μ g of DNA vaccine that was administered to the other two groups of mice after the first DNA immunization. Twenty-eight days after the third and final immunization, the mice were sacrificed and plasma samples were stored at -20°C until they were analyzed. The control groups of mice received only vector DNA (formulated like the experimental groups) that did not contain the gene for the envelope protein of HIV-2_{UC2}. Sero-conversion and reciprocal antibody titers were determined using a baculovirus expressed recombinant HIV-2_{ST} gp120 polypeptide (a generous gift from Dr. Margery Chaikin, SmithKlineBeecham, King of Prussia, PA). An optical density value of 0.2 was used to define the reciprocal end-point antibody titer. The results were summarized and analyzed using nonparametric methods since the distribution of the data was non-Gaussian. The significance of differences between two groups was calculated with the Spearman Rank Test using the StatView software program (Abacus Concepts, Cary, NC).

RESULTS AND DISCUSSION

To evaluate the various adjuvants for genetic immunization, we compared naked DNA immunization with DNA contained within an Alzet minipump, or formulated with chitosan or cationic liposomes (Vaxfectin). The HIV-2 gp140-specific antibodies were evaluated 28 days after each immunization. After the third immunization, serum from the group of mice immunized with naked DNA and the Alzet minipump had similar end-point antibody titers (a mean of approximately 1/400; Fig. 1A). The group of mice immunized with HIV-2 *env* DNA formulated with Vaxfectin had a 3.5 increase in their end-point antibody titers (1/1750; $P = 0.027$) while the group of mice immunized with HIV-2 *env* DNA formulated with chitosan had lower antibody titers (1/200).

In the measurement of IgG-specific subclasses, we found that the group of mice immunized with naked DNA had a predominant IgG1 subclass, while mice immunized with Vaxfectin had the highest level of IgG2a and IgG2b than any of the other groups of mice (Fig. 1B; $P = 0.03$). In addition, none of the groups of mice had a predominant shift in the IgG1:IgG2a ratio, indicating that the DNA formulations did not shift the T-helper responses to either a Th1 or Th2-type bias.

To evaluate the levels of HIV-2-specific mucosal antibodies, we tested saliva and vaginal mucosal wash samples in each of the groups of mice. Similar to serum antibodies, the group of mice immunized with Vaxfectin had the highest levels of total IgG, A, and M and the highest levels of IgA in vaginal washes compared with naked DNA, DNA delivered in an Alzet minipump or chitosan (Fig. 2; $P = 0.034$).

To study the effect of the route of immunization using DNA formulated with Vaxfectin, we next immunized groups of mice by the intradermal, intramuscular, and intranasal routes. After three immunizations, the antibody levels were evaluated by end-point ELISA titers. We found that immunization by the intradermal route gave the highest levels of HIV-2-specific serum antibodies (end-point titer of $>1/3200$ for total IgG) compared to intramuscular immunization (end-point titer of 1/2700) while immunization by the intranasal route gave the lowest levels (end-point titer of 1/700). In contrast, the levels of HIV-specific mucosal antibodies were highest in the group of mice im-

munized by the intramuscular route ($>1/16$ versus 1/8 for intradermal and intranasal).

In this study, the group of mice immunized by the intradermal route with a DNA vaccine formulated with Vaxfectin had the highest serum and mucosal antibody titers compared to the other groups that we evaluated. Vaxfectin may function as a DNA adjuvant by acting as a depot adjuvant and protecting the DNA from nuclease degradation. Since DNA complexed with cationic lipids appears to be immunostimulatory (Dow *et al.*, 1999), these complexes may also activate the innate immune response by signaling antigen-presenting cells through their Toll-like receptors and/or CD1 molecules by their phospholipid moieties. How cationic liposome adjuvant formulations for DNA vaccines influence antigen-specific immune responses when compared to nonionic block copolymers and PLGA cationic microparticle adjuvant formulations remains to be determined.

Chitosan is primarily used for the oral and nasal administration of whole inactivated and recombinant protein-based vaccines as well as DNA vaccines (Illum *et al.*, 2001; MacLaughlin *et al.*, 1998; McNeela *et al.*, 2000). It may be that chitosan works best as an adjuvant when it is used as a delivery formulation of DNA vaccines by the oral and nasal routes and not by the intradermal route. The osmotic mini pump was found to not substantially boost the immune responses in mice. Although one study has reported its use with a recombinant polypeptide derived envelope vaccine from HIV-1 (Cleland *et al.*, 1996), the effect on the immune response of an osmotic delivery pump to immunize with DNA was unknown. These results suggest that bolus injections of DNA, rather than a slow controlled release (in this case, 20 μ g/28 days), was more effective for inducing high titers of HIV specific antibodies.

In summary, priming with DNA vaccines using cationic lipid formulations such as Vaxfectin may work synergistically in prime/boost immunization modalities. The optimization of vaccine strategies for HIV will be critical to more effectively reduce HIV transmission and provide better therapeutic immunization strategies that prevent the development of AIDS.

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Address reprint requests to:
Christopher P. Locher, Ph.D.
Department of Infectious Diseases
Maxygen, Inc.
515 Galveston Drive
Redwood City, CA 94063

E-mail: christopher.locher@maxygen.com

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